

Article

Construction and Expression of a Single Chain Antibody Mimicking Human Ovarian Cancer Antigen CA125

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One concept for immune therapy of cancer involves induction of antigen mimic antibodies to trigger the immune response against tumor cells. Anti-idiotypic antibodies directed against the antigen-binding site of antibodies specific for tumor antigen may functionally and even structurally mimic antigen and induce anti-anti-idiotypic immune response. Monoclonal antibody WJ02 is one of such anti-idiotypic antibodies, which contains internal image of CA125. In order to improve the immunospecificity of mAb WJ02, we constructed a single chain of mAb WJ02 in V1-linker-Vh orientation. The scFv-WJ02 could be expressed and secreted in the recombinant *Pichia pastoris* system. The secreted scFv protein with a molecular weight of 30 kD retained the biological activity of mAb WJ02, which was proved by a direct binding assay and inhibition experiment. Our results indicated that the scFv-WJ02 could be used as a possible tool for idiotypic therapy against ovarian cancer, which might enhance the possibility of eliminating nonspecific responses induced by mAb WJ02. *Cellular & Molecular Immunology*. 2006; 3(1):59-62.

Key Words: anti-idiotypic, single chain antibody, *P. pastoris*

Introduction

Induction of tumor-specific immunity with nominal tumor antigens has achieved limited success when the antigens are not readily available or when the host is tolerant to the nominal antigens. CA125 is such an antigen with these characteristics, which is expressed by 80% of ovarian carcinomas. Since the CA125 gene has not been cloned, the antigen is not available for human immunization. Therefore, activation of the idiochrome network using Ab2 mimicking CA125 offers an alternative approach to immunotherapy of CA125-expressing carcinomas (1-4).

According to the immune network theory, idiotopes at the antigen-combining site of an antibody can elicit anti-idiotypic antibodies (Ab2). Among polyclonal Ab2s, there are subsets bearing the internal image of the antigen epitopes

(Ab2 β). Ab2 β s in turn can induce anti-anti-idiotypic antibodies (Ab3) that recognize the original antigen expressed by an infectious agent or cancer cells. Thus using Ab2 β s as surrogate antigens for cancer vaccine represents one of the most promising approaches for active immunotherapy.

WJ02 is a monoclonal antibody raised against the murine monoclonal immunoglobulin MJ01, which defines ovarian cancer antigen CA125 (5). It had been proved that this antibody has a homology to the CA125 sequence by inducing humoral and cellular responses *in vivo* (3).

In the current study, the single chain of mAb WJ02 was constructed and expressed. Single chain antibody scFv-WJ02 is composed of V1 and Vh domains of mAb WJ02, which has the same protein construction as the epitopes on CA125. Therefore, scFv-WJ02 can function in a similar manner as nominal antigen. The strategies used to produce scFv proteins have primarily relied on bacterial expression, such as in *Escherichia coli*. However, in most cases, the production of scFv as insoluble inclusion bodies with the bacterial cytoplasm has proved to be of limited use due to the problems of the refolding process and functional product recovery. In this study, a methylotrophic yeast *Pichia pastoris* expression system was used, which can overproduce a variety of eukaryotic proteins with high secretion-efficiency (6). The gene encoding the variable region of mAb WJ02 was inserted in the place of the *P. pastoris* alcohol oxidase (AOX1) gene and the expression of the cloned gene is thus under the control of the strong and methanol inducible AOX1 gene promoter. The anti-idiotypic activity of scFv-

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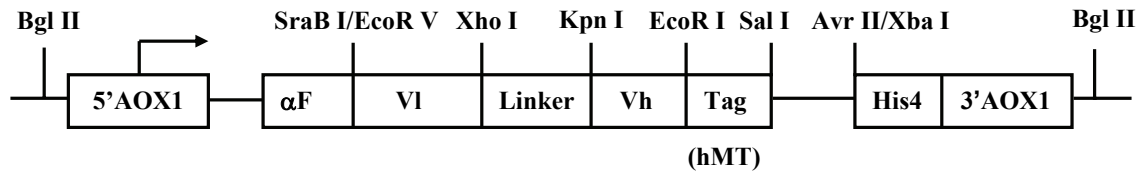


Figure 1. Plasmid construction of scFv-WJ02 with VI-linker-Vh orientation controlled by inducible AOX1 gene promoter. The plasmids derived from vector pPIC-9 contained α F secretion signal.

WJ02 was further investigated by binding assay and inhibition experiment. We demonstrate here the successful construction of functional scFv-WJ02 in *P. pastoris* yeast system.

Materials and Methods

Bacteria and yeast strains

E. coli TOP10F was used for all plasmid constructions. *P. pastoris* GS115 used for expression of scFv and GS115/albumin as a positive control of secretion was obtained from Invitrogen. All *P. pastoris* cells were cultured in buffered minimal glycerol medium at 30°C. For the induction of protein expression in recombinant *P. pastoris* cells, buffered minimal methanol medium was used.

Construction of scFv plasmid

Anti-idiotypic antibody WJ02 was generated as described previously (3). The variable domain sequences were PCR-amplified using sequence specific primers, and engineered into a cloning vector with VI-linker-Vh orientation, resulting in plasmid pWJ02. A tag was designed to locate at the C-terminus, containing a human metallothionein (hMT) sequence which could be used as a radioactive metal binding domain. The *P. pastoris* expression/secretion vector, pPIC-9, obtained from Invitrogen, was used to construct recombinant scFv-WJ02 expression plasmid. The 2,453 bp Pvu II/Xba I fragment containing the scFv-WJ02 coding sequence was inserted into the SnaB I/Avr II sites of the *P. pastoris* vector pPIC-9 (with the α F secretion signal). Therefore, the cloned scFv had the VI-linker-Vh orientation and hMT as the tag (Figure 1).

Transformation of *P. pastoris* by electroporation

The plasmid DNAs were transformed into competent GS115 cells by electroporation using a Gene Pulser (Bio-Rad) and the resulting transformants were then plated for His⁺ selection onto Regeneration Dextrose Base agar. The colonies were screened by replica plating onto both glycerol- and methanol-containing plates. The colonies with retarded growth rate on the methanol-containing plate had a His⁺, mut⁻ (methanol utilization deficient) phenotype and, therefore, were selected for protein expression.

Iodination of single chain antibody

Five hundred micrograms of scFv-WJ02 were labeled with

20 mCi Na¹²⁵I using Iodobeads (Pierce). Following a 30-min incubation period at room temperature, free radioisotope was removed by Sephadex G25 gel filtration using PBS as the elution buffer. The labeling efficiency was 97%.

Protein expression, purification and characterization

The selected clones were isolated and cultured in induction medium. After a three-day induction, the cell supernatants were collected and then analyzed for protein expression by SDS-PAGE (Figure 2). Proteins were subsequently purified using HPLC. Ab1-binding specificity was determined by immunoradioactive assay, in which the ability of iodinated scFv-WJ02 to bind mAb MJ01 coated solid phase was detected.

Inhibition of Ab1 binding to CA125 by scFv-WJ02

The microtiter plates were coated by CA125 (10 U/ml) and blocked by 3% BSA/PBS. A mixture of mAb MJ01 (1 μ g/ml) and scFv-WJ02 was added to these wells. The concentrations of scFv-WJ02 ranged from 0 to 10 μ g/ml (0, 1, 2, 4, 8, 10 μ g/ml). The plates were incubated at room temperature for 1 h. The plates were then washed three times with PBS, and bound mAb MJ01 was detected by incubation with peroxidase-labeled goat anti-mouse H and L antibody (Southern Bio. Assoc.) for 1 h at room temperature (100 μ l/ml). Following three washes, 100 μ l of ABTS Peroxidase Substrate solution was added. The absorbance was measured at 405 nm wavelength.

Results and Discussion

It is well established in several systems that monoclonal anti-Id can potentially play a role in vaccine development, by virtue of their ability to mimic the nominal antigen and to stimulate the immune system (7-9). Since the studies in murine tumor systems have clearly shown that Ab2 can induce specific and protective immunity, a number of anti-Ids have been generated in goats, mice and rabbits that mimic the original antigens (10-12). In the current study, a single chain antibody, scFv-WJ02, was designed as a cancer vaccine agent for this purpose. Single chain antibody scFv-WJ02 is composed of VI and Vh domains of mAb WJ02, which is an anti-idiotypic antibody that mimics human ovarian cancer-associated antigen CA125. Single chain antibody has many significant advantages over whole immunoglobulins for the purpose of targeted immunotherapy.

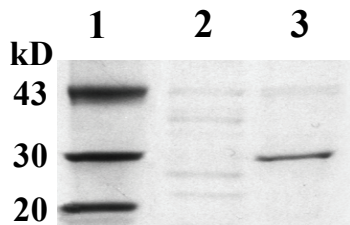


Figure 2. SDS-PAGE of scFv-WJ02 on reducing gel. Lane 1, protein marker; Lane 2, culture media after HPLC purification; Lane 3, scFv-WJ02.

Since only the variable region of anti-idiotypic antibody reflects the image of antigen, vaccination using single chain antibody should reduce the non-specific immune responses. Therefore, the clinical application of scFv-WJ02 for anti-idiotypic therapy should eliminate HAMA responses, which limit the use of repeated cycles of antibody therapy.

*Expression and secretion of scFv-WJ02 in recombinant *P. pastoris**

The goal of the study was to construct and express a functional scFv in the *P. pastoris* system. The DNA fragments coding for the engineered scFv-WJ02 with V1-linker-Vh-tag orientation was constructed into *P. pastoris* vector, pPIC-9 with α F secretion signals. After linearization by Bgl II digestion, the plasmid DNAs were transformed into competent GS115 cells by electroporation and the resulting transformants were selected on histidine-deficient media. After screening for integration at the correct loci, only colonies growing on a his^r/glycerol⁺ plate but proliferating in a slow speed on a his^r/methanol⁺ plate were isolated, cultured in induction media, and analyzed for protein expression by SDS-PAGE. Our results indicated that the V1-linker-Vh orientation of scFv-WJ02 could be successfully expressed and secreted in the *P. pastoris* system. The yield of scFv-WJ02 was approximately 35 mg per liter of culture medium. The purity of scFv-WJ02 produced in *P. pastoris* system was greater than 95% as illustrated by SDS-PAGE (Figure 2). Under the reducing conditions, scFv-WJ02 showed the expected molecular weight of 30 kD.

Biological activities of scFvWJ02 protein

Desplancq et al. demonstrated that scFvs in the V1-linker-Vh format showed greater binding activities than other orientation (13). In order to demonstrate that scFv-WJ02 proteins secreted from the recombinant *P. pastoris* cells have their intrinsic binding activity, the anti-idiotypic binding properties of scFv-WJ02 were first evaluated in a direct binding assay (Figure 3). The result showed that scFv-WJ02 reacted with mAb MJ01 in a dose-dependent manner, which indicates that scFv-WJ02 retained the binding ability of the original antibody to the determinants presented on the variable region of mAb MJ01. The specificity of binding to the variable region of Ab1 was further confirmed by an inhibition assay (Figure 4). When coincubated with scFv-

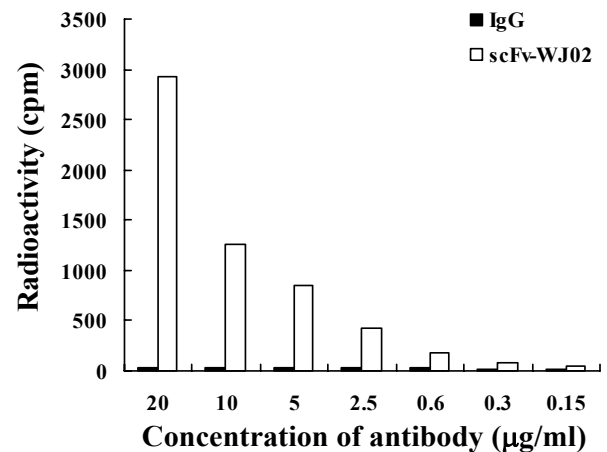


Figure 3. Anti-idiotypic binding activity of scFv-WJ02. Anti-CA125 antibody mAb MJ01 was coated on a solid phase and targeted by ¹²⁵I-labeled scFv-WJ02. The binding ability was determined by the radioactivity remained on the solid phase. The unrelated mouse IgG was used as a control. The binding efficiency was dependent on the concentration of scFv-WJ02.

WJ02, the binding of mAb MJ01 to CA125 was greatly abolished. At the concentration of 4 µg/ml, the single chain antibody could inhibit 50% of the binding of mAb MJ01 to CA125. The inhibition had been proved to be specific previously by the original antibody WJ02 (3). Our previous experience showed that only the V1-linker-Vh orientation of the engineered scFv could be successfully expressed and secreted in the *P. pastoris* system (14). Single chain antibody in this format requires a linker less than 25 amino acids between V1 and Vh to retain greater binding activity than those with the Vh-linker-V1 sequence, because the C-terminus of Vh and/or the N-terminus of V1 make structural

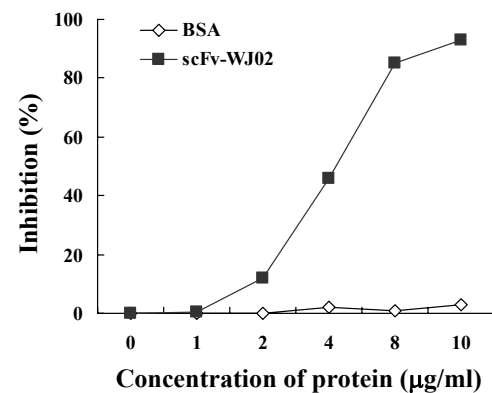


Figure 4. The inhibition of Ab1 binding to CA125 by scFv-WJ02. Certain amount of CA125 was coated on the microtiter plates. The binding of mAb MJ01 to CA125 was determined by ELISA. scFv-WJ02 interfered this binding in a dose dependent manner. BSA was used as negative control.

contributions to the binding. In this study, a linker of 15 amino acids was inserted between V_L and V_H.

In conclusion, the constructed scFv-WJ02 protein retained the biological activities of mAb WJ02, which mimics CA125 and induces humoral and cellular immune responses against ovarian cancer. The high level of expression achieved in *P. pastoris* system can readily provide very large quantities of the single chain protein used as a potential cancer therapeutic agent in the future.

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